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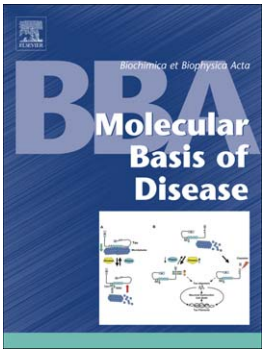

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METABOLIC, ENZYMATIC AND GENE INVOLVEMENT IN CEREBRAL GLUCOSE DYSMETABOLISM AFTER TRAUMATIC BRAIN INJURY

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Running title: Glucose dysmetabolism in graded TBI

ABSTRACT

In this study, the metabolic, enzymatic and gene changes causing cerebral glucose dysmetabolism following graded diffuse traumatic brain injury (TBI) were evaluated. TBI was induced in rats by dropping 450g from 1 (mild TBI; mTBI) or 2 m height (severe TBI; sTBI). After 6, 12, 24, 48, and 120 h gene expressions and enzymatic activities of glycolysis and pentose phosphate pathway (PPP) enzymes, and levels of lactate, ATP, ADP, ATP/ADP (indexing mitochondrial phosphorylating capacity), NADP+, NADPH and GSH were determined in whole brain extracts (n = 6 rats at each time for both TBI levels). Sham-operated animals (n = 6) were used as controls. Results demonstrated that mTBI caused a late increase (48-120 h post injury) of glycolytic gene expression and enzymatic activities, concomitantly with mitochondrial functional recovery (ATP and ATP/ADP normalization). No changes in lactate and PPP genes and enzymes, were accompanied by transient decrease in GSH, NADP+, NADPH and NADPH/NADP+. Animals following sTBI showed early increase (6-24 h post injury) of glycolytic gene expression and enzymatic activities, occurring during mitochondrial malfunctioning (50% decrease in ATP and ATP/ADP). Higher lactate and lower GSH, NADP+, NADPH, NADPH/NADP+ than controls were recorded at anytime post injury ($p < 0.01$). Both TBI levels caused metabolic and gene changes affecting glucose metabolism. Following mTBI, increased glucose flux through glycolysis is coupled to mitochondrial glucose oxidation. "True" hyperglycolysis occurs only after sTBI, where metabolic changes, caused by depressed mitochondrial phosphorylating capacity, act on genes causing net glycolytic flux increase uncoupled from mitochondrial glucose oxidation.

Key words: Traumatic brain injury; glucose dysmetabolism; glycolysis; pentose phosphate pathway; energy metabolism; mitochondrial dysfunction.

1. INTRODUCTION

Physiologically, the brain supports its energy demand by using glucose as its main metabolic fuel (1). To ensure maximal energy yield, glucose must be fully oxidized through mitochondrial/oxygen dependent reactions (2), since glycolysis contributes to ATP generation with a modest 5.26% and mitochondria with 94.74%. Hence, to sustain the cerebral energy demand, glucose consumption must be coupled to mitochondrial functions and oxygen consumption, i.e. oxidative phosphorylation must generate 3 or 2 ATP/pair of electrons transferred from NADH or $FADH_2$ to $½O_2$ via the electron transfer chain (ETC)[1]. Pathologically, cerebral energy state imbalance can occur for two reasons: i) hypoxia/ischemia, when decrease in the cerebral blood flow (CBF) decreases oxygen delivery to the tissue and mitochondria (3-5); ii) mitochondrial dysfunction, when the stoichiometric ratio - moles of transferred electrons through ETC/moles of O_2 reduced to water/moles of ATP produced by complex V – is impaired (6, 7). The profound distinction between these two phenomena is that the latter occurs under adequate CBF and tissue oxygen delivery (8). Decrease in mitochondrially generated ATP during cerebral energy dysmetabolism is partly counterbalanced by increasing the glycolysis rate (9-11).

[1]**Abbreviations used in the text are:** CBF, cerebral blood flow; ETC, electron transfer chain; TBI traumatic brain injury; mTBI, mild traumatic brain injury; sTBI, severe traumatic brain injury; PPP, pentose phosphate pathway; PEP, phosphoenolpyruvate; 1,3-BPG, 1,3-bisphosphoglycerate; G-6-PDH, glucose-6-phosphate dehydrogenase; 6-PGDH, 6-phosphogluconate dehydrogenase; TIM, triosephosphate isomerase; GDH, glycerol-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; LOD, lactate oxidase; B2M, β-2-microglobulin; HK, hexokinase; PFK, phosphofructokinase; PGK, phosphoglycerokinase; PK, pyruvatekinase.

Experimental and clinical evidences demonstrated that traumatic brain injury (TBI), the leading cause of death and disability under 45 years of age in Western countries (12), provokes cell energy state derangement (13), mitochondrial dysfunction (14, 15) and ATP decrease (13, 16). Mitochondrial dysfunction with energy crisis may be transient, as in mild TBI (mTBI) (13), or permanent, as in severe TBI (sTBI) (13, 15). Several studies evidenced brain energy dysregulation even in TBI patients (10, 17-20). Hyperglycolysis, i.e. the increased glucose consumption rate through glycolysis with no parallel increase in mitochondrial (oxygen) dependent glucose oxidation, and stimulation of the pentose phosphate pathway (PPP) were reported as common features following TBI (21-24). Both phenomena occur early post injury and are protracted for a not well-defined period after TBI (21, 25). Currently, no information about the timing of glucose dysmetabolism, influence of injury severity and changes in the gene expression and activity of the glycolytic and PPP enzymes are available.

Here, we investigated the gene expressions and activities of glycolytic and PPP enzymes, as well as the levels of lactate, ATP, ADP, ATP/ADP ratio, $NADP^+$, NADPH and GSH, at different times following graded diffuse TBI in rats, highlighting a multifactorial metabolic, enzymatic and gene contributions affecting glucose and energy metabolism in the post-injured brain.

2. MATERIALS AND METHODS

2.1 Experimental protocol

The experimental protocol was approved by the Ethical Committee of the Catholic University of Rome, in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals. Male Wistar rats of 300-350g were randomly divided into: 1) sham-operated as control; 2) mild diffuse TBI (mTBI group); 3) severe diffuse TBI (sTBI group). As for the anesthetic mixture, animals received 35 mg/kg b.w. ketamine and 0.25 mg/kg b.w. midazolam by i.p. injection. TBI was induced by dropping a 450 g weight from 1m (mTBI) or 2m height (sTBI), according to the weight drop impact acceleration model (26). Rats that suffered from skull fracture, seizures, nasal bleeding, or did not survive the impacts (n = 6 in the sTBI group), were discarded and not included in the study. At 6, 12, 24, 48, and 120 h from injury, rats (n = 9 for each time point in both groups of injured animals) were again anesthetized and then immediately sacrificed. Sham-operated animals sacrificed 120 h after the initial anesthesia (n = 9) were used as controls.

2.2 Tissue preparation for the determination of gene expressions, enzymatic activities and metabolites

As described in detail elsewhere (27), an in vivo craniectomy was performed in all animals during anesthesia and the two hemispheres were freeze-clamped in liquid nitrogen to minimize metabolite loss (28, 29). Total RNA was extracted by homogenizing one hemisphere in Trizol (Invitrogen Life Technologies), using the Ultra-Turrax homogenizer (Janke Kunkel, Staufen, Ge) at 24,000 rpm/min, to produce a final 10% homogenate (weight:volume). Crude homogenates suitable to measure enzymatic activities, were obtained by homogenizing one hemisphere in 15 mM KCl + 1 mM KH_2PO_4, pH 7.4, at 24,000 rpm/min for 90 sec in the cold, followed by centrifugation at 18,690 x g for 15 min at 4 °C.

The tissue preparation for the simultaneous HPLC analysis of ATP, ADP, GSH, $NADP^+$ and NADPH was performed on one hemisphere, using the organic solvent deproteinization described elsewhere (30). As previously indicated (29, 31), the utilization of these protocols for tissue manipulation and the proper mixing of the different processing of the right and left hemispheres, allowed the simultaneous determination of the gene expressions (three right + three left hemispheres), enzyme activities (three right + three left hemispheres) and quantification of metabolites (three right + three left hemispheres), by using nine animals for each time point.

2.3 Analysis of gene expressions of the main glycolytic and PPP enzymes

Transcription to cDNA of RNA extracted from brain samples and subsequent real time-quantitative polymerase chain reaction (RT-qPCR) were performed as previously described (29, 31). Primers, reported in Table 1, were designed with the 0.2 version of the Primer3 Input software developed by the Whitehead Institute for Biomedical Research (Cambridge, MA) and using as templates the sequences of *Rattus norvegicus* published by the National Center for Biotechnology Information. For accurate gene expression measurements with RTqPCR, results were normalized to the housekeeping genes of beta-2-microglobulin (B2M, NM_012512) and Ubiquitin C (Ubc NM_017314.1) of *Rattus norvegicus*, selected from twelve candidate reference genes using the geNorm Housekeeping Gene Selection Kit (Primer Design Ltd.). Changes in transcript abundance of tested genes were calculated using the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (32).

2.4 Determination of the activities of selected glycolytic and PPP enzymes

The activities of the glycolytic enzymes hexokinase (HK), phosphofructokinase (PFK), phosphoglycerokinase (PGK), pyruvatekinase (PK) and lactate dehydrogenase (LDH), and of the PPP enzymes glucose-6-phosphate dehydrogenase (G-6-PDH) and 6-phosphogluconate

dehydrogenase (6-PGDH) were determined on 100 μl of 10% homogenates, prepared as above described. In the case of HK, PFK PK, LDH, G-6-PDH and 6-PGDH the reactions were carried out at 37 °C according to standardized protocols (33). The change in absorbance of $NAD(P)^+$ or NADH was followed spectrophotometrically at 340 nm wavelength, using an Agilent 89090A spectrophotometer (Agilent Technologies, Santa Clara Ca, USA). In the case of PGK, the amount of the reaction product was measured by HPLC. Briefly, a reaction mixture containing (in final concentrations) 20 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 1.5 mM 1,3-BPG, 4 mM ADP and 100 μl sample was incubated at 37 °C. Before and after 5, 10 and 20 minutes of 1,3-BPG supplementation, aliquots of the mixture were withdrawn, deproteinized (30) and then loaded onto the HPLC column to measure the amount of ATP produced. In brain extracts processed for the enzymatic analysis, proteins were measured according to the Bradford method (34). Enzyme activities were expressed as mU/mg protein. In the case of HK, PFK, PK, LDH, G-6-PDH and 6-PGDH calculations were performed using 6.3×10^{-3}, as the millimolar extinction coefficient of $NADP^+$ or NAD(P)H at 340 nm wavelength.

2.5 Analyses of metabolites

The spectrophotometric lactate determination was carried out on 50 μl of brain tissue extracts following the lactate oxidase method described by Artiss et al. (35). The simultaneous separation and quantification of ATP, ADP, $NADP^+$, NADPH and GSH in deproteinized brain extracts (20 μl), or ATP quantification in deproteinized PGK reaction mixtures (200 μl), was performed by high performance liquid chromatography (HPLC), according to methods formerly set up in our laboratory (30, 36, 37).

2.6 Statistical analysis

Normal data distribution was tested using the Kolmogorov-Smirnov test. The within-group comparison at each time was performed by the one-way analysis of variance (ANOVA). Differences across groups were estimated by the two-way ANOVA for repeated measures. Fisher's protected least square was used as the post hoc test. Only two-tailed p-values of less than 0.05 were considered statistically significant.

3. RESULTS

A mortality rate of 11.8% (6/51 rats) was recorded in the group of sTBI, whilst all mTBI rats survived to the impact for the desired time. This grossly accounts for the different severity of injury using this model of TBI.

3.1 Effect of graded TBI on the gene expression and enzymatic activity of HK

During 24 h post injury, mTBI rats showed no changes in the HK1gene expression (Fig. 1A). At 48 and 120 h post impact, the HK1 gene expression increased by 2.3 and 3.5 fold ($p < 0.001$ respect to controls). Conversely, sTBI rats showed a very rapid 1.8 and 3.2 fold increase at 6 and 24 h post injury ($p < 0.01$ respect to both controls and corresponding times of mTBI). A tendency to normalization at 48 and 120 h after injury, when HK1 overexpression in sTBI rats was significantly lower than values determined in mTBI animals ($p < 0.01$), was then recorded. The HK enzymatic activity in mTBI rats (Fig. 1B) increased by 1.4 and 2 fold only at 48 and 120 h post trauma ($p < 0.01$ compared to controls). In sTBI rats HK activity (Fig. 1B) showed a significant 1.9 and 2 fold increase at 24 and 48 h ($p < 0.01$ compared to both controls and mTBI animals). However, at 120 h after injury HK values were similar to those of controls, but significantly lower than those found in mTBI rats ($p < 0.01$).

3.2 Effect of graded TBI on the gene expression and enzymatic activity of PFK

Similarly to HK1, the gene expression of PFKL (Fig. 1C) did not change shortly (6 and 24 h) after an mTBI. Prolonging the time of recovery to 48 and 120 h produced a striking 3.8 and 6.2 times overexpression of the PFKL gene ($p < 0.01$ respect to controls) (Fig. 1C). Differently, the PFKL gene expression in sTBI animals showed a bell-shaped curve with a progressive increase over the control value by 2.3 and 5.7 times at 6 and 24 h post injury ($p <$

0.01 respect to both controls and mTBI rats). At 48 and 120 h post sTBI, the PFKL expression tended to normalize and at 120 h it was 2.5 lower than the value measured in mTBI animals ($p < 0.01$). The PFK enzymatic activity almost mirrored the changes in the gene expression of PFKL (Fig. 1D). Therefore, mTBI produced a significant increase in PFK activity at 48 and 120 h ($p < 0.01$ compared to controls). Severely injured animals had a maximum of PFK activity at 24 h post impact ($p < 0.01$ compared to both controls and mTBI rats), hereinafter showing a slow tendency to normalization. Again, after 120 h sTBI animals had PFK activity lower (1.5 times) than that measured in mTBI rats ($p < 0.01$).

3.3 Effect of graded TBI on the gene expression and enzymatic activity of PGK

The gene expression of PGK1 in mTBI injured rats (Fig. 2,A) progressively increased by 2.4, 4.1 and 5.2 times at 24, 48 or 120 h from impact ($p < 0.001$ respect to controls). In sTBI rats, the PGK1 gene was overexpressed at any time after injury ($p < 0.001$ compared to controls), with a trend to normalize at 48-120 h post injury (Fig. 2A). Again, sTBI rats exhibited, at 120 h post trauma, a 2.5 fold lower expression of PGK than mTBI animals ($p < 0.01$). The enzymatic activity of PGK (Fig. 2B) was significantly higher than that of controls at 48 (+152%, $p < 0.01$) and 120 h (+262%, $p < 0.01$), in rats receiving an mTBI; conversely, in sTBI rats (Fig. 2B) PGK progressively increased during the initial 24 h followed by a return towards pre-impact values hereinafter. At 120 h after sTBI, PGK activity was equal to that of controls but lower that that of mTBI rats ($p < 0.01$).

3.4 Effect of graded TBI on the gene expression and enzymatic activity of PK

The gene expression of PKM in mTBI rats (Fig. 2C) was significantly overexpressed only at 48 and 120 h from impact (1.8 and 2.2 times higher than controls $p < 0.01$). In sTBI rats, the PKM gene was overexpressed at 6 and 24 h post injury (1.7 and 2.1 times higher than the value of controls, $p < 0.01$) with no differences from controls at 48 and 120 h post impact

(Fig. 2C). At this time point, PKM expression in sTBI rats was almost 3 orders of magnitude lower than that of mTBI animals ($p < 0.01$). The enzymatic activity of PK (Fig. 2D) was significantly higher than that of controls only at 48 and 120 h post mTBI ($p < 0.01$). In sTBI rats (Fig. 2D) PK activity was higher than both controls and mTBI injured animals only at 24 h after trauma ($p < 0.01$). At 120 h after injury PK activity of sTBI animals was equal to that of controls but almost 3 times lower that that of mTBI rats ($p < 0.01$).

3.5 Effect of graded TBI on the gene expression and enzymatic activity of LDH

Figures 3A and 3B illustrate the changes in the expressions of the genes controlling the synthesis of the two subunits of the tetrameric enzyme LDH, i.e. LDHA and LDHB. The expression of both genes was evaluated since neurons and astrocytes contain, in different proportions, the two omotetrameric LDH isoforms composed by four identical A or B type subunits. In mTBI animals, no change of LDHA was observed at any time post injury (Fig. 3A), while significant increase in LDHB expression ($p < 0.01$ compared to controls) was recorded at 120 h after trauma (Fig. 3B). Following sTBI, expressions of both LDHA (Fig. 3A) and LDHB (Fig. 3B) significantly increased compared to both controls and mTBI rats ($p < 0.05$). In consequences of this differential gene expression, activity of total LDH (Fig. 3C) increased only at 120 h in mTBI rats ($p < 0,05$ respect to both controls and sTBI animals) and at 48 h in sTBI rats ($p < 0,05$ respect to both controls and mTBI animals).

3.6 Effect of graded TBI on the gene expression of additional glycolytic enzymes

Changes in the gene expression of aldolase (ALDOC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and neuronal specific enolase (ENO2) are reported in Table 2. As occurred to the other glycolytic enzymes, the post TBI gene expressions of ALDOC, GAPDH and ENO2 was influenced by either the severity of injury or the time interval after impact. In mTBI rats, the gene expression of the three enzymes significantly increased respect to

controls at 48 and 120 h post trauma ($p < 0.05$), with GAPDH and ENO2 showing the most evident changes. When considering these gene expressions in sTBI animals, it can be observed that ALDOC was significantly increased at 24 and 48 h post injury and GAPDH at 24 h only, whilst ENO2 did not show any significant variation during our observational period.

3.7 Effect of graded TBI on the enzymatic activity of G-6-PDH

It is worth recalling that it was not possible to measure the expression of the G6PDH gene since the sequence of the *Rattus norvegicus*, nor of the *Mus musculus* gene is available. Therefore, only the G-6-PDH activity was assessed. In mTBI animals, no significant changes in G-6-PDH activity at any time after trauma were observed (Fig. 4A). Conversely, a 2 and 1.5 fold increase, respectively at 24 and 48 h post impact ($p < 0.05$ compared to both controls and mTBI rats), was recorded in rats receiving an sTBI.

3.8 Effect of graded TBI on the gene expression and enzymatic activity of 6-PGDH

The expression of 6-PGDH in mTBI rats did not vary at any time point after injury (Fig. 4B), whilst severe injury caused 6-PGDH to overexpress slightly only after 24 and 48 h ($p < 0.05$ compared to both controls and mTBI rats). Similarly to G-6-PDH, mTBI did not provoke any change in the activity of 6-PGDH (Fig. 4C), which modestly increased only after 24 and 48 h after sTBI ($p < 0.05$ compared to both controls and mTBI rats).

3.9 Effect of graded TBI on the gene expression of additional PPP enzymes

Table 2 indicates that the gene expression of TKT (transketolase) was not affected by either mTBI or sTBI. The expression of TALDO1 (transaldolase) did not change in mTBI rats but underwent a slight but significant downregulation in animals receiving an sTBI ($p < 0.05$ compared to controls).

3.10 Effect of graded TBI on brain lactate, energy metabolism and mitochondrial functions

In Figure 5, the time course changes of lactate, ATP, ADP and ATP/ADP ratio in rats following mTBI or sTBI are reported. Rats experiencing an mTBI, did not show significant changes in whole brain lactate at any time post injury (Fig. 5A). Conversely, sTBI caused brain lactate to increase from 1.83 μmol/g wet weight (value of controls) to 4.76 μmol/g wet weight at 6 h post impact ($p < 0.01$ compared to both controls and mTBI rats). A slow decline in cerebral lactate levels occurred in sTBI rats at 24 (3.76 μmol/g wet weight) and 48 h (3.22 μmol/g wet weight). At 120 post impact, lactate levels were still +44% higher than the value of controls (2.64 μmol/g wet weight, $p < 0.01$ compared to both controls and mTBI animals). The time course of ATP concentrations (Fig. 5B) was characterized by a biphasic trend in mTBI animals, with an initial decrease (minimum of ATP recorded at 24 h post injury) followed by spontaneous recovery up to complete normalization within 48-120 h. Severely injured rats showed a remarkable ATP decrease early after impact (-31% at 6 h, $p < 0.01$ respect to both controls and mTBI rats), followed by no recovery even at 120 h post sTBI, when ATP was only the 65% of the control value ($p < 0.01$ compared to both controls and mTBI rats). Following an mTBI, no changes in ADP concentration were recorded (Fig. 5C). However, cerebral ADP levels increased in sTBI animals, with significantly higher values at any time post injury ($p < 0.01$ compared to controls and mTBI rats at corresponding times). When calculating the ATP/ADP ratio (Fig. 5D), which is considered an index of the mitochondrial phosphorylating capacity (38), it was observed that this progressively declined during the first 24 h post injury in mTBI rats (-35% decrease recorded at 24 h; $p < 0.01$ compared to controls) and spontaneously recovered afterwards, up to reach the pre-impact values at 120 h after injury. Conversely, the ATP/ADP ratio in sTBI rats sharply declined by 53% at 6 h from trauma ($p < 0.01$) and subsequently remained lower by about 60% than the

control value ($p < 0.01$), thus suggesting a permanent impairment of the ETC-coupled oxidative phosphorylation, i.e. the main mitochondrial function.

3.11 Effect of graded TBI on GSH homeostasis

To evaluate whether the TBI-induced changes in the gene expressions and activities of the two NADPH-generating enzymes of the PPP (G-6-PDH and 6-PGDH) were connected to GSH homeostasis, we measured GSH, $NADP^+$ and NADPH, and we calculated the NADPH/$NADP^+$ ratio, at the different times following graded injury. Data indicate (Fig. 6A) that mTBI caused a transient decrease in GSH cerebral content at 24 and 48 h post injury, when this antioxidant decreased by 20 and 15% ($p < 0.05$ compared to controls). Conversely, GSH in sTBI was significantly lower than that of controls at any time point ($p < 0.01$). It is worth highlighting that at 120 h a 46% decrease in GSH occurred in sTBI animals ($p < 0.01$ compared to both controls and mTBI rats). $NADP^+$ (Fig. 6B) NADPH (Fig. 6C) and NADPH/$NADP^+$ (Fig. 6D) did not change at any time following mTBI. On the contrary, a steady decline of these parameters was recorded in sTBI animals with minimal values determined at 120 h post impact ($p < 0.01$ compared to both controls and mTBI rats).

4. DISCUSSION

We have here demonstrated that the cerebral glucose dysmetabolism following TBI, causing profound changes in energy and redox balance, does not only depend on the alterations of the complex regulatory mechanisms of metabolic pathways and cycles (1, 39) but it is also influenced by overexpression of genes encoding for glycolytic enzymes and for selected PPP enzymes.

Using a graded diffuse (non focal) TBI model (26), we showed that the expressions of the genes encoding for the four kinases of glycolysis, namely HK, PFK, PGK and PK, undergo time-dependent and severity-dependent changes, causing differential changes of their respective activities. When considering the HK1, PFKL, PGK1 and PK genes, as well as the corresponding enzymatic activities (HK, PFK, PGK and PK), the most striking difference between mTBI and sTBI was the very different timing in their overexpression or change in activity, occurring within 120 h from injury.

In mildly injured rats, whilst no changes occurred during the first 24 h, a 3 to 5 fold increases in the expressions of these genes took place between 48 and 120 h post impact (Fig. 1 and 2), with the consequent increase in the corresponding enzyme activities, mainly occurring 120 h after trauma (Fig. 1 and 2). This pattern was also observed for the glycolytic ALDOC, GAPDH and ENO2 genes (Table 2), thus confirming a differential timing following mTBI or sTBI. These results are connected to the variations of whole brain lactate, energy metabolites (ATP and ADP) and mitochondrial phosphorylating capacity (ATP/ADP ratio), occurring after an mTBI (Fig 5), indicating a transient impairment of the main mitochondrial function of producing adequate ATP (decrease in the ATP/ADP ratio) to satisfy the cell energy demand (15, 16, 27). Under these conditions, the brain activates a gene neuroprotection program, a sort of hibernating strategy, based on the rationalization of energy production and consumption (40). The aim is to reduce the risk of increasing reactive oxygen and nitrogen

species, produced/mediated by malfunctioning mitochondria and capable of irreversibly damaging biochemically essential molecules (41, 42). Therefore, during the initial 24-36 h post injury, relatively modest changes in metabolites and activation of a proper gene strategy avoid relevant changes in the glycolytic gene expressions and enzymatic activities. In coincidence with the mitochondrial function normalization (recovery of ATP/ADP ratio), occurring between 48 and 120 h, glycolysis has to support an increased demand of substrates for TCA cycle, ETC and oxidative phosphorylation aimed to restore ATP homeostasis (Fig. 5). We found that during this period of time (48-120 h post mTBI) gene expressions and activities of the four kinases of glycolysis increased 3 to 5 times, clearly leading to an increased rate of glucose utilization through glycolysis. The concomitant restoration of mitochondrial functions ensured an overall higher glucose consumption coupled to oxygen consumption, required to restore ATP to pre impact levels. Therefore, increase of gene expressions and activities of glycolytic enzymes occurring in mTBI are related to the recovery phase of energy metabolism. This should not be considered as hyperglycolysis but as "hyper glucose oxidative metabolism" aimed to support the increased energy demand needed for the simultaneous energy replenishment and ongoing cell repair processes (43). The lack of increase in whole brain lactate, and of LDH expression and activity, observed in our experiments, corroborates this hypothesis, suggesting that mTBI triggers signalling mechanisms not involving lactate and acting as positive regulators of the expression of glycolytic genes in the metabolic recovery phase post injury.

Changes in the four kinases following sTBI (Figures 1 and 2) showed bell-shaped curves peaking at 24 (gene expressions) or 48 h (enzymatic activities). Early post sTBI (6 h), cerebral tissue underwent a sudden 35% ATP decrease and a dramatic 55% reduction of the ATP/ADP ratio, accompanied by a 260% rise in lactate, indicating an immediate glucose dysmetabolism caused by mitochondrial impairment (Fig. 5). The increase in lactate and ATP catabolites (AMP, oxypurines, nucleosides) (13, 44, 45), signalling a marked post injury

energy imbalance, mirrored the gene expression increase of HK1, PFKL, PGK1, PK, LDHA and LDHB, followed by an increase of the corresponding enzyme activities. This type of adaptive response can be interpreted as an attempt to speed up the glycolytic rate, as this remains the main pathway to supply ATP during severe mitochondrial malfunctioning. Notwithstanding, this metabolic and gene driven hyperglycolysis failed to affect positively the cerebral energy state because of the minimal energetic yield of glycolysis (no increase in ATP at any time post sTBI). Steady high values of whole brain lactate indicate that the combined metabolic and gene stimulation of glycolysis produced an increased rate of glycolytically consumed glucose. This occurred during severe mitochondrial malfunctioning (very low levels of the ATP/ADP ratio), i.e. when pyruvate produced by glycolysis cannot be further metabolized by TCA and is reduced to lactate by increased LDH (LDHA and LDB overexpressions). Therefore, these results provide a persuasive explanation that the lactate increase, reported in several studies (10, 20, 46-49), is caused by the "true" sTBI-induced hyperglicolysis.

The expressions and activities of the PPP genes and enzymes seem to indicate a close relationship with GSH and NADP(H) metabolism, again highlighting profound differences between mTBI and sTBI. Whilst G-6PDH and 6-PGDH did not change in mTBI animals, increase in gene expression and activity of both dehydrogenases occurred 24-48 h after sTBI. However, even in sTBI, neither TKT nor TALDO1 were overexpressed (Table 2), thus suggesting that the goal was not to promote a general PPP increase, but rather to activate selectively its NADPH-generating reactions. The alterations of GSH, NADPH, $NADP^+$, and NADPH/$NADP^+$ ratio indicate the occurrence of a sustained oxidative/nitrosative stress (50, 51), causing a transient GSH depletion after an mTBI and a drastic, progressive decrease after an sTBI. This confirms the hypothesis that partial PPP activation in severely injured animals is connected to GSH homeostasis (25, 46). In sTBI, the increase of the two NADPH-

producing reactions limited the decrease in the NADPH/$NADP^+$ ratio despite a drastic depletion of this pyridine nucleotide pool (Fig. 6).

Summarizing, it appears that the post injured brain, under the pressure of energy imbalance, adopts a selected modulation of the genes controlling the expressions of glycolytic and PPP pathways, which depends on injury severity. Considering the time course of lactate, energy metabolites and mitochondrial phosphorylating capacity, it appears that the resulting increase in the glycolytic flux in mTBI is concomitant with the mitochondrial function recovery, i.e. glucose consumption is coupled and coincident with glucose oxidation. Additionally, minimal or no modifications of the PPP pathway indicate no metabolic switch of glucose metabolism aimed to bypass glycolysis. When considering sTBI, it is evident that the net increase in the glucose flux through glycolysis (increase in lactate) takes place while mitochondrial metabolism is profoundly depressed, i.e. glucose consumption is not coupled and coincident with glucose oxidation. The glycolytic gene overexpression, particularly of the four kinases, occurred relatively early after injury and might be mediated by metabolite changes consequent to the sudden and drastic deterioration in the energy state and mitochondrial phosphorylating capacity. Together with the marked lactate increase, these metabolic changes most likely acted as signals for specific gene targets (glycolytic genes). Therefore, it appears that sTBI produces a "true" hyperglycolytic state aimed to counteract the progressive ATP decrease in presence of persistent mitochondrial dysfunction. As even after an sTBI we did not observe an overall PPP activation, but a selective activation of its NADPH-generating reactions, we can conclude that this is rather linked to maintaining GSH homeostasis (25, 46) as opposed to an actual metabolic switch in glucose metabolism (21, 39).

In conclusion, in this experimental model, a genuine glucose metabolism dysregulation occurs only after sTBI, involving deep metabolic alterations acting at the gene level and leading to hyperglycolysis during persistent mitochondrial malfunctioning. Conversely, after an mTBI positive modulations in the glycolytic gene expressions also occurs, but these are

coincident with mitochondrial functional recovery and do not constitute "true" hyperglycolysis. The differential biochemical and molecular strategies and their time courses adopted by a mildly or a severely injured brain to regulate glucose metabolism have to be considered when developing new pharmacological approaches targeting glucose dysmetabolism after TBI. Furthermore, it would be highly advisable, either in experimental or clinical studies, to specify clearly what severity of injury the results refer to. It is more and more evident that the word TBI is merely descriptive of a physical insult. Whether this insult does or does not evolve into a multifaceted pathology, characterized by complex mechanisms of cerebral cell dysregulation, is strictly dependent on the severity of injury.

5. ACKNOWLEDGEMENTS

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7. **FIGURE LEGENDS**

Figure 1. Time course changes of HK1 (Panel A) and PFKL (Panel C) gene expressions, and enzymatic activities of HK (Panel B) and PFK (Panel D), in rats receiving mTBI or sTBI. Controls are represented by a group of sham operated rats (n = 9). Values at each time point are the mean of 6 animals (3 left + 3 right hemispheres). Standard deviations are represented by vertical bars. Gene expressions were calculated relatively to β-2-microglobulin (B2M). One enzymatic unit, relative to protein, corresponds to 1 μmol of substrate/min/mg protein transformed by the enzyme.

*significantly different from controls, $p < 0.05$

**significantly different from corresponding time of mTBI rats, $p < 0.05$.

Figure 2. Time course changes of PGK1 (Panel A) and PKM (Panel C) gene expressions, and enzymatic activities of PGK (Panel B) and PK (Panel D), in rats receiving mTBI or sTBI. Controls are represented by a group of sham operated rats (n = 9). Values at each time point are the mean of 6 animals (3 left + 3 right hemispheres). Standard deviations are represented by vertical bars. Gene expressions were calculated relatively to β-2-microglobulin (B2M). One enzymatic unit, relative to protein, corresponds to 1 μmol of substrate/min/mg protein transformed by the enzyme.

*significantly different from controls, $p < 0.05$

**significantly different from corresponding time of mTBI rats, $p < 0.05$

Figure 3. Time course changes of LDHA (Panel A) and LDHB (Panel B) gene expressions, and enzymatic activity of total LDH (Panel C), in rats receiving mTBI or sTBI. Controls are represented by a group of sham operated rats (n = 9). Values at each time point are the mean of 6 animals (3 left + 3 right hemispheres). Standard deviations are represented by vertical bars. Gene expressions were calculated relatively to β-2-microglobulin (B2M). One enzymatic unit, relative to protein, corresponds to 1 μmol of substrate/min/mg protein transformed by the enzyme.

*significantly different from controls, $p < 0.05$

**significantly different from corresponding time of mTBI rats, $p < 0.05$

Figure 4. Time course changes of enzymatic activity of G-6-PDH (Panel A), of 6-PGDH gene expression (Panel B), and enzymatic activity of 6-PGDH (Panel C), in rats receiving mTBI or sTBI. Controls are represented by a group of sham operated rats (n = 9). Values at each time point are the mean of 6 animals (3 left + 3 right hemispheres). Standard deviations are represented by vertical bars. Gene expression was calculated relatively to β-2-microglobulin (B2M). One enzymatic unit, relative to protein, corresponds to 1 μmol of substrate/min/mg protein transformed by the enzyme.

*significantly different from controls, $p < 0.05$

**significantly different from corresponding time of mTBI rats, $p < 0.05$.

Figure 5. Time course changes of cerebral concentrations of ATP (Panel A), ADP (Panel B), and of the ATP/ADP ratio (Panel C), in rats receiving mTBI or sTBI. Controls are represented by a group of sham operated rats (n = 9). Values of the parameters at each time point are the mean of 6 animals (3 left + 3 right hemispheres). Standard deviations are represented by vertical bars.

* significantly different from controls, $p < 0.05$

**significantly different from corresponding time of mTBI rats, $p < 0.05$

Figure 6. Time course changes of cerebral concentrations of GSH (Panel A), $NADP^+$ (Panel B), NADPH (Panel C) and of the NADPH/$NADP^+$ ratio (Panel D), in rats receiving mTBI or sTBI. Controls are represented by a group of sham operated rats (n = 9). Values of the parameters at each time point are the mean of 6 animals (3 left + 3 right hemispheres). Standard deviations are represented by vertical bars.

* significantly different from controls, $p < 0.05$

** significantly different from the corresponding time in mTBI rats, $p < 0.05$.

Table 1. Sequences of primers used to evaluate the expressions of the gene regulating the synthesis of the main glycolytic and pentose phosphate pathway enzymes.

Gene symbol	Gene sequence	Forward	Reverse
HK1	NM_012734.1	GCTCAGAAAAGGGGGATTTC	GATGTTCTCTGGGGTGTCGT
PFKL	NM_013190.4	CAACTGGCTCAGTGTCTCCA	GACACACAGGTTGGTGATGC
ALDOC	NM_012497.1	GCCTCTAGCTGGGACTGATG	GCGATCACTGATTTTCAGCA
GAPDH	NM_017008.4	TGCCACTCAGAAGACTGTGG	TTCAGCTCTGGGATGACCTT
PGK1	NM_053291.3	ACAACATGGAGATTGGCACA	GTAGCTTGGCCAGTCTTTGC
ENO2	NM_139325.3	GTGGACCACATCAACAGCAC	TCTCAGTCCCATCCAACTCC
PKM	NM_053297.2	TTTCCAATCCTGCATTCCTC	CCTATCCATTAGGCCAGCAA
LDHA	NM_017025.1	TGTGACTGCAAACTCCAAGC	GAAGCCGCTGATCTTCCAAG
LDHB	NM_012595.1	ACTCCGTGACAGCCAATTCT	CGAAACCGAGCAGAATCCAG
6PGDH	NM_001305435.1	ACTCTTCACTCCTCTGGTGC	ACGTGAACATCCAAGTGTGC
TKT	NM_022592.1	TGTCACCAAGGGCTAGGAAG	CTTTCCACTCGGTACACCCT
TALDO1	NM_031811.3	TCACCATCTCACCCAAGCTT	AGAGCTTCTCCACAGCCATT

Table 2. Changes in the expression of the genes encoding for the glycolytic enzymes aldolase (ALDOC), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and enolase (ENO2), as well as for the PPP enzymes transketolase (TKT) and transaldolase (TALDO1), following mTBI or sTBI.

	ALDOC/B2M	**GAPDH/B2M**	**ENO2/B2M**	**TKT/B2M**	**TALDO1/B2M**
Control	1.00 ± 0.07	1.02 ± 0.28	1.03 ± 0.29	1.02 ± 0.29	1.07 ± 0.11
mTBI 6 h	1.07 ± 0.06	0.59 ± 0.12	1.22 ± 0.23	0.77 ± 0.23	0.89 ± 0.18
mTBI 24 h	1.02 ± 0.23	1.21 ± 0.21	1.36 ± 0.27 [a]	0.85 ± 0.27	1.14 ± 0.20
mTBI 48 h	1.53 ± 0.22[a]	1.29 ± 0.16	1.74 ± 0.40 [a]	1.05 ± 0.40	0.85 ± 0.21
mTBI 120 h	1.51 ± 0.31[a]	1.94 ± 0.19 [a]	2.34 ± 0.37 [a]	0.86 ± 0.37	0.96 ± 0.27
sTBI 6 h	1.01± 0.24	0.57 ± 0.14	0.72 ± 0.09	0.87 ± 0.19	0.67 ± 0.09[a]
sTBI 24 h	1.31 ± 0.34	1.50 ± 0.35 [a]	1.03 ± 0.11	0.89 ± 0.21	0.69 ± 0.12[a]
sTBI 48 h	1.58 ± 0.18[a]	1.01 ± 0.19	0.88 ± 0.25	0.78 ± 0.25	0.78 ± 0.25
sTBI 120 h	1.16 ± 0.06	0.74 ± 0.25	0.89 ± 0.21	0.69 ± 0.21[a]	0.69 ± 0.21[a]

Values are the means ± standard deviations of six different animals. Gene expressions are reported as the ratios with the housekeeping gene B2M.

[a]significantly different from controls, $p < 0.05$.

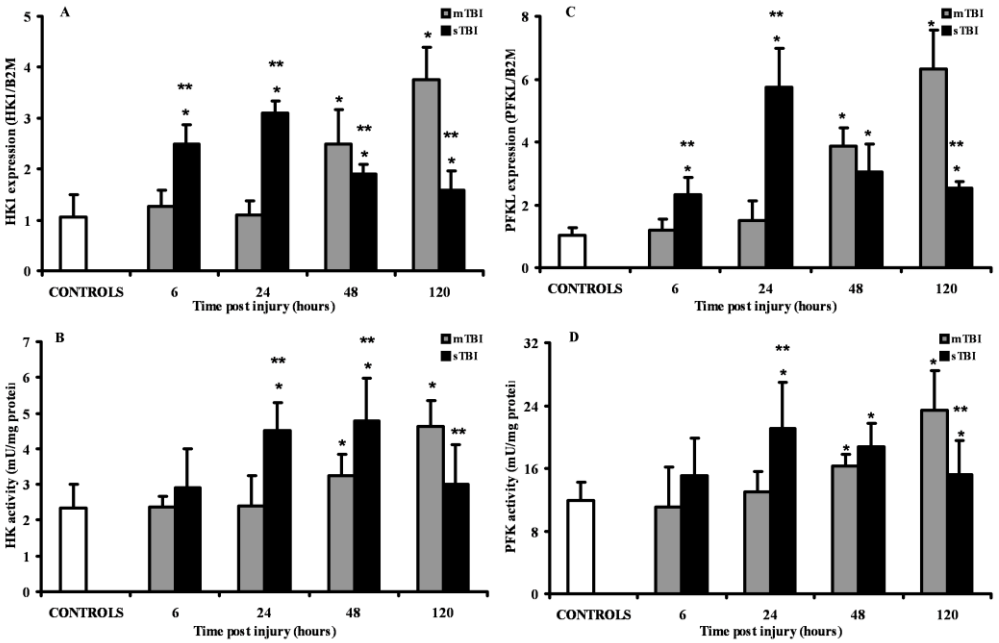

Fig. 1

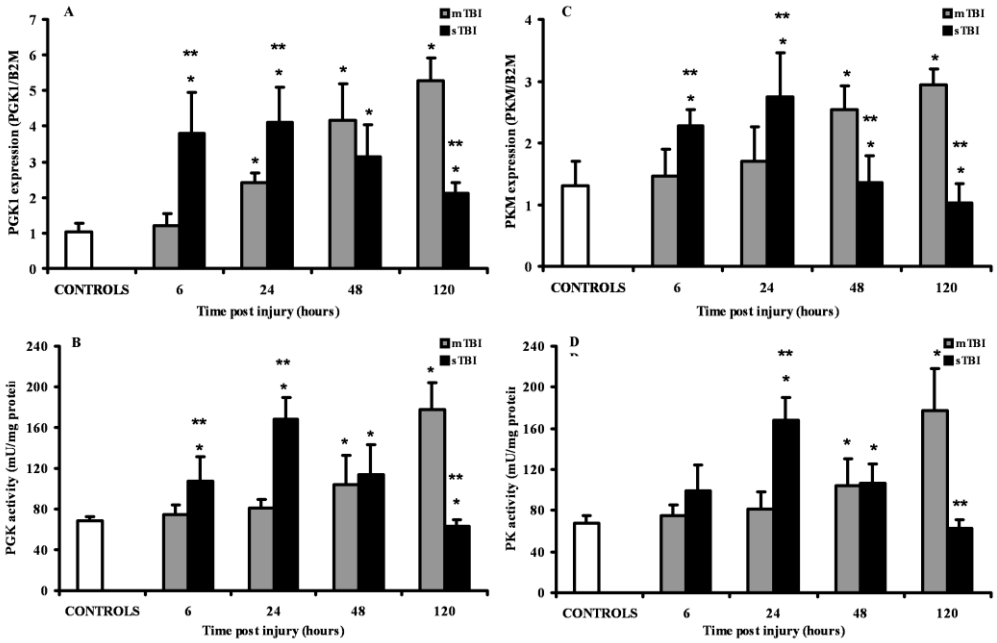

Fig. 2

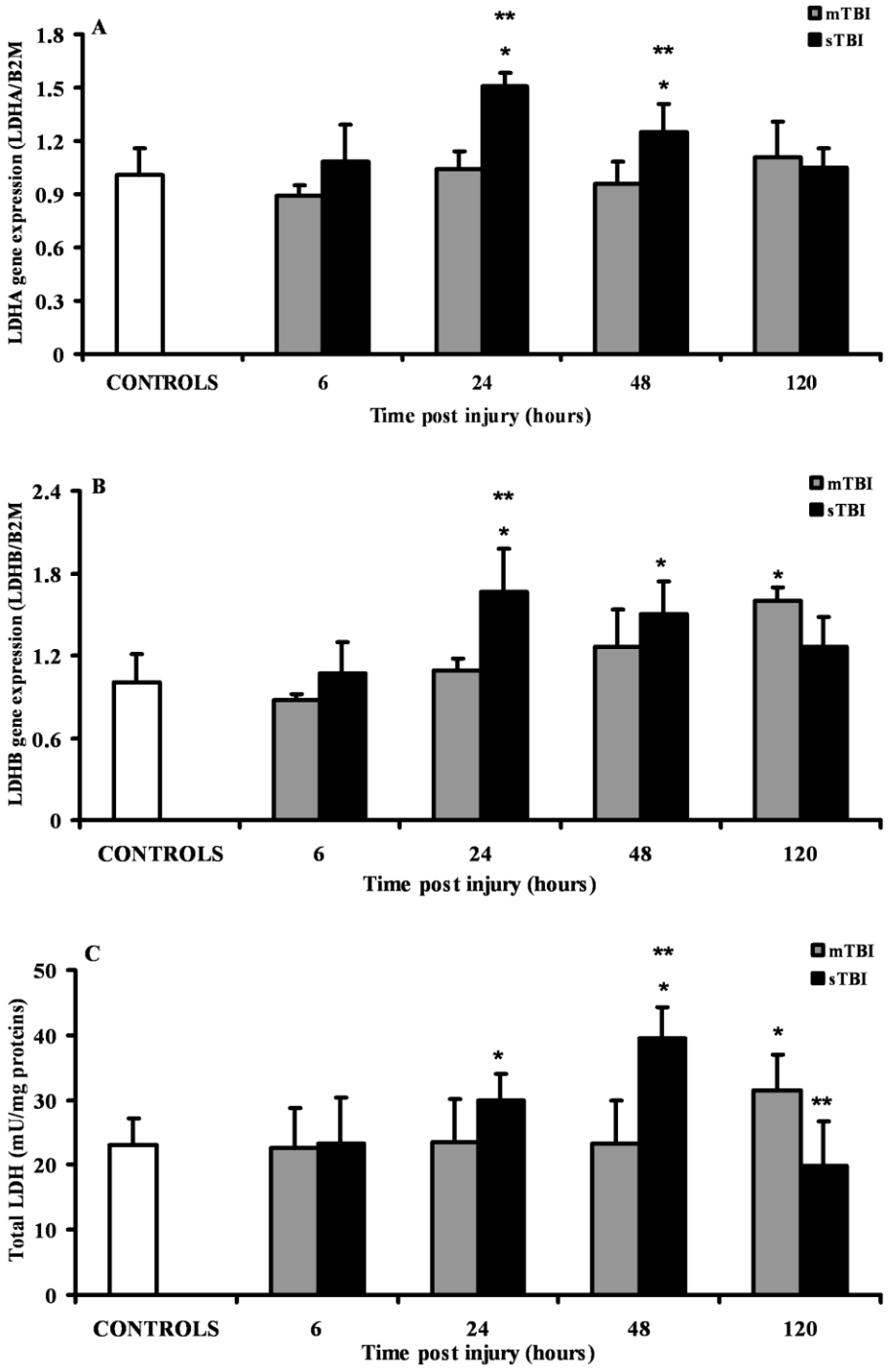

Fig. 3

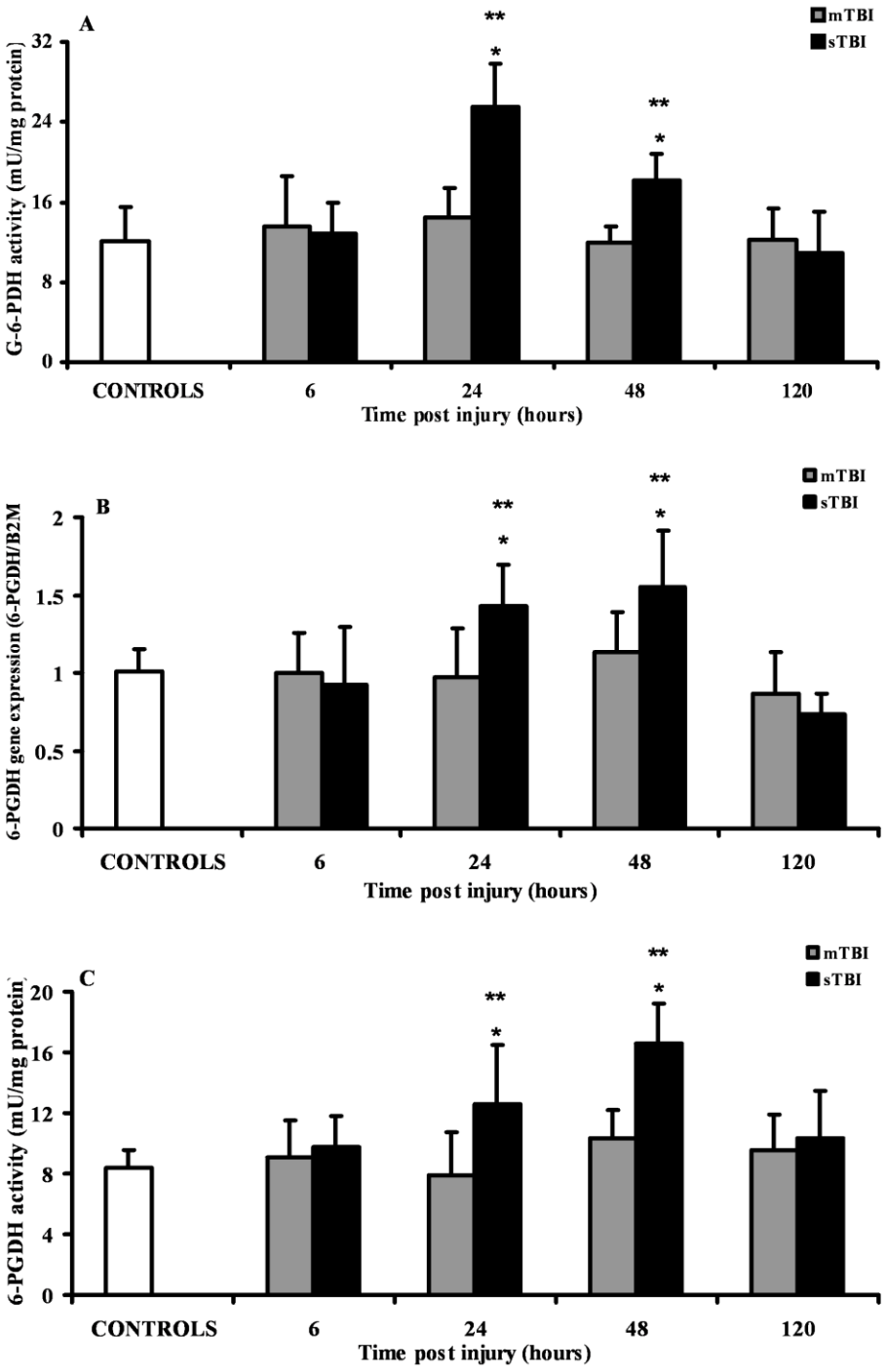

Fig. 4

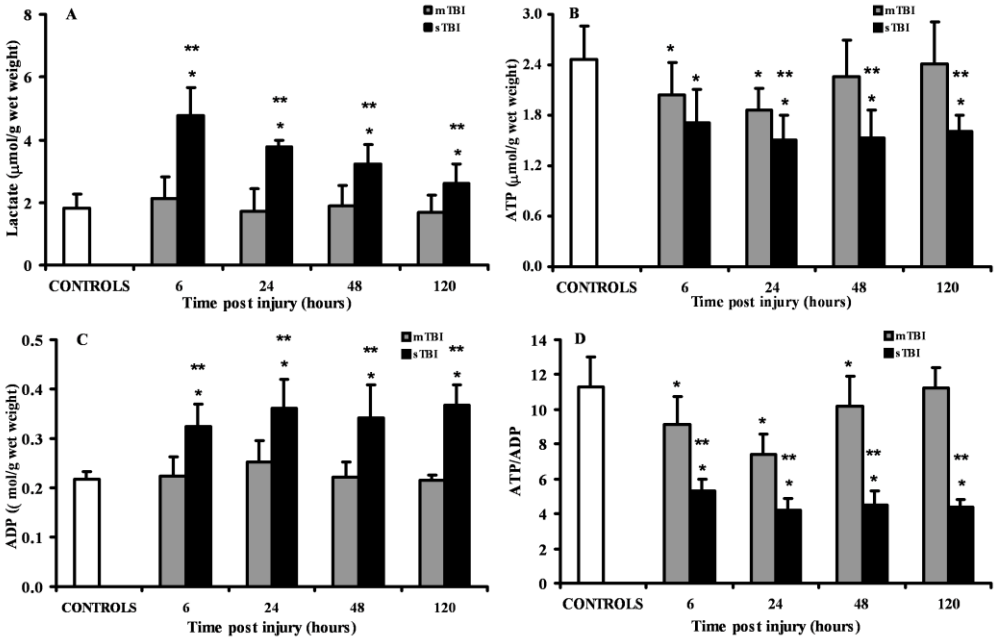

Fig. 5

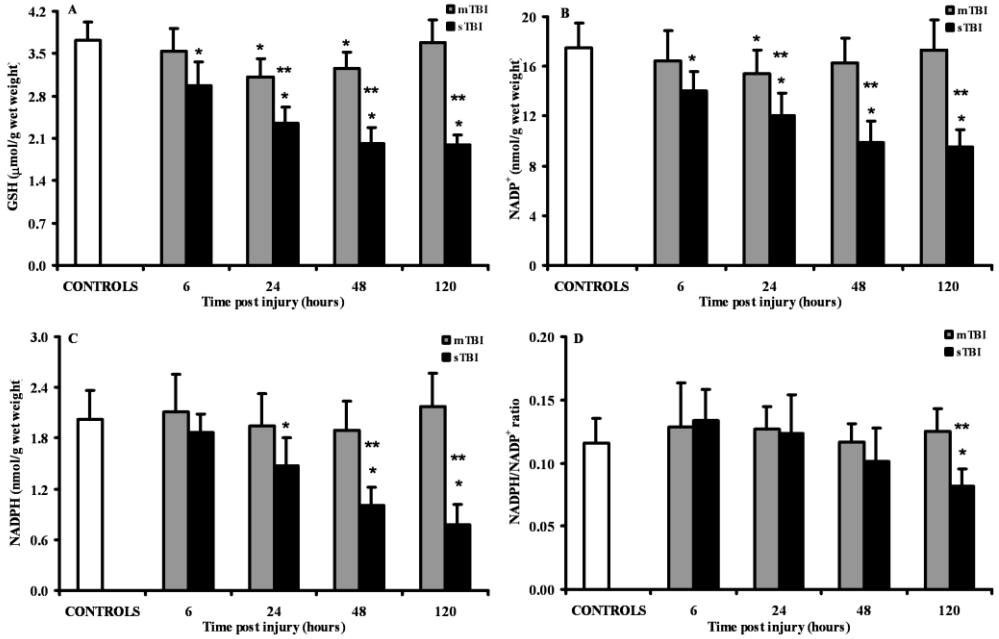

Fig. 6

Highilights

- Metabolic, enzymatic and gene changes are involved in glucose dysmetabolism after TBI
- Activation of glycolysis in mild TBI occurs on recovery of mitochondrial functions
- Activation of glycolysis in severe TBI occurs during mitochondrial malfunctioning
- Pentosephosphate pathway changes in severe TBI are limited to NADPH supply
- Metabolic and gene mechanisms of glucose dysregulation depend on TBI severity